CRYSTALS OF GLUCOKINASE AND METHODS OF GROWING THEM

PRIORITY TO RELATED APPLICATIONS

[0001] This application is a Divisional of Serial No. 10/318,308, filed December 12, 2002, which is now pending. This application claims the benefit of U.S. Provisional Application(s) Serial No. 60/341,988, filed December 19, 2001.

FIELD OF THE INVENTION

[0002] The invention relates to crystalline forms of Gluckokinase of sufficient size and quality to obtain structural data by X-ray crystallography and to methods of growing such crystals.

BACKGROUND OF THE INVENTION

[0003] Glucokinase (GK) is one of four hexokinases found in mammals [Colowick, S.P., in *The Enzymes*, Vol. 9 (P. Boyer, ed.) Academic Press, New York, NY, pages 1-48, 1973]. The hexokinases catalyze the first step in the metabolism of glucose, i.e., the conversion of glucose to glucose-6-phosphate. Glucokinase has a limited cellular distribution, being found principally in pancreatic β-cells and liver parenchymal cells. In addition, GK is a rate-controlling enzyme for glucose metabolism in these two cell types that are known to play critical roles in whole-body glucose homeostasis [Chipkin, S.R., Kelly, K.L., and Ruderman, N.B. in *Joslin's Diabetes* (C.R. Khan and G.C. Wier, eds.), Lea and Febiger, Philadelphia, PA, pages 97-115, 1994]. The concentration of glucose at which GK demonstrates half-maximal activity is approximately 8 mM. The other three hexokinases are saturated with glucose at much lower concentrations (<1 mM). Therefore, the flux of glucose through the GK pathway rises as the concentration of

glucose in the blood increases from fasting (5 mM) to postprandial ($\approx$10-15 mM) levels following a carbohydrate-containing meal [Printz, R.G., Magnuson, M.A., and Granner, D.K. in *Ann. Rev. Nutrition* Vol. 13 (R.E. Olson, D.M. Bier, and D.B. McCormick, eds.), Annual Review, Inc., Palo Alto, CA, pages 463-496, 1993]. These findings contributed over a decade ago to the hypothesis that GK functions as a glucose sensor in β-cells and hepatocytes (Meglasson, M.D. and Matschinsky, F.M. *Amer. J. Physiol.* **246**, E1-E13, 1984). In recent years, studies in transgenic animals have confirmed that GK does indeed play a critical role in whole-body glucose homeostasis. Animals that do not express GK die within days of birth with severe diabetes while animals overexpressing GK have improved glucose tolerance (Grupe, A., Hultgren, B., Ryan, A. et al., *Cell* **83**, 69-78, 1995; Ferrie, T., Riu, E., Bosch, F. et al., *FASEB J.*, **10**, 1213-1218, 1996). An increase in glucose exposure is coupled through GK in β-cells to increased insulin secretion and in hepatocytes to increased glycogen deposition and perhaps decreased glucose production.

[0004] The finding that type II maturity-onset diabetes of the young (MODY-2) is caused by loss of function mutations in the GK gene suggests that GK also functions as a glucose sensor in humans (Liang, Y., Kesavan, P., Wang, L. et al., *Biochem. J.* **309**, 167-173, 1995). Additional evidence supporting an important role for GK in the regulation of glucose metabolism in humans was provided by the identification of patients that express a mutant form of GK with increased enzymatic activity. These patients exhibit a fasting hypoglycemia associated with an inappropriately elevated level of plasma insulin (Glaser, B., Kesavan, P., Heyman, M. et al., *New England J. Med.* **338**, 226-230, 1998). While mutations of the GK gene are not found in the majority of patients with type II diabetes, compounds that activate GK and, thereby, increase the sensitivity of the GK sensor system will still be useful in the treatment of the hyperglycemia characteristic of all type II diabetes. Glucokinase activators will increase the flux of glucose metabolism in β-cells and hepatocytes, which will be coupled to increased insulin secretion. Such agents would be useful for treating type II diabetes.

[0005] In an effort to elucidate the mechanisms underlying kinase activation , the crystal structure of such proteins is often sought to be determined. The crystal structures of several hexokinases have been reported. See, e.g. A. E. Aleshin, C. Zeng, G. P. Bourenkov, H. D. Bartunik, H. J. Fromm & R. B. Honzatko 'The mechanism of regulation of hexokinase: new insights from the crystal structure of recombinant human brain hexokinase complexed with glucose and glucose-6-phosphate' *Structure* **6**, 39-50 (1998); W. S. Bennett, Jr. & T. A. Steitz 'Structure of a complex between yeast hexokinase A and glucose I. Structure determination and refinement at 3.5 Å resolution' *J. Mol. Biol.* **140**, 183-209 (1978); and S. Ito, S. Fushinobu, I. Yoshioka, S. Koga, H. Matsuzawa & T. Wakagi 'Structural Basis for the ADP-Specificity of a Novel Glucokinase from a Hyperthermophilic Archaeon' *Structure* **9**, 205-214 (2001). Despite these reports, researchers armed with the knowledge of how to obtain crystals of related hexokinases have attempted to obtain crystals of any mammalian Glucokinase without success.

SUMMARY OF THE INVENTION

[0006] Applicants have discovered protocols which allow crystallization of mammalian Glucokinase with or without a bound allosteric ligand. The crystal structure has been solved by X-ray crystallography to a resolution of 2.7 Å. See Figures 3 and 4. Thus the invention relates to a crystalline form of Gluckokinase and a crystalline form of a complex of Glucokinase and an allosteric ligand. The invention further relates to a method of forming crystals of Glucokinase, with or without a bound allosteric ligand.

BRIEF DESCRIPTION OF THE FIGURES

[0007] Figure 1 shows Glucokinase co-crystals having P6(5)22 symmetry.

[0008] Figure 2 shows the amino acid sequence of an expressed Glucokinase used for crystallization.

[0009]   Figure 3 shows a ribbon diagram of the structure of Glucokinase colored according to secondary structure. Light blue represents α-helix, dark blue represents 3_{10}-helix, green represents β-sheet and orange is coil.

[0010]   Figure 4 shows the atomic structure coordinates for Glucokinase bound to 3-Cyclopentyl-2-pyridin-4-yl-N-thiazol-2-yl-propionamide.

DETAILED DESCRIPTION OF THE INVENTION

[0011]   The present invention relates to crystalline forms of mammalian Glucokinase, with or without a ligand bound in the allosteric site, where the crystals are of sufficient quality and size to allow for the determination of the three-dimensional X-ray diffraction structure to a resolution of about 2.0 Å to about 3.5 Å. The invention also relates to methods for preparing and crystallizing the Glucokinase. The crystalline forms of Glucokinase, as well as information derived from their crystal structures can be used to analyze and modify glucokinase activity as well as to identify compounds that interact with the allosteric site.

[0012]   The crystals of the invention include apo crystals and co-crystals. The apo crystals of the invention generally comprise substantially pure Glucokinase. The co-crystals generally comprise substantially pure Glucokinase with a ligand bound to the allosteric site.

[0013]   It is to be understood that the crystalline Glucokinases of the invention are not limited to naturally occurring or native Glucokinases. Indeed, the crystals of the invention include mutants of the native Glucokinases. Mutants of native Glucokinases are obtained by replacing at lest one amino acid residue in a native Glucokinase domain with a different amino acid residue, or by adding or deleting amino acid residues within the native polypeptide or at the N- or C- terminus of the native polypeptide, and have substantially the same three-dimensional structure as the native Glucokinase from which the mutant is derived.

[0014] By having substantially the same three-dimensional structure is meant having a set of atomic structure coordinates from an apo- or co-crystal that have a root mean square deviation of less than or equal to about 2 Å when superimposed with the atomic structure coordinates of the native Glucokinase from which the mutant is derived when at least about 50% to about 100% of the alpha carbon atoms of the native Glucokinase are included in the superposition.

[0015] In some instances, it may be particularly advantageous or convenient to substitute, delete and/or add amino acid residues to a native Glucokinase domain in order to provide convenient cloning sites in cDNA encoding the polypeptide, to aid in purification of the polypeptide, etc. Such substitutions, deleteions and/or additions which do not substantially alter the three dimensional structure of the native Glucokinase will be apparent to those having skills in the art.

[0016] It should be noted that the mutants contemplated herein need not exhibit glucokinase activity. Indeed, amino acid substitutions, additions or deletions that interfere with the kinase activity of the glucokinase but which do not significantly alter the three-dimensional structure of the domain are specifically contemplated by the invention. Such crystalline polypeptides, or the atomic structure coordinates obtained therefrom, can be used to identify compounds that bind to the native domain. These compounds may affect the activity or the native domain.

[0017] The derivative crystals of the invention generally comprise a crystalline glucokinase polypeptide in covalent association with one or more heavy metal atoms. The polypeptide may correspond to a native or a mutated Glucokinase. Heavy metal atoms useful for providing derivative crystals include, by way of example and not limitation, gold and mercury. Alternatively, derivative crystals can be formed from proteins which have heavy atoms incorporated into one or more amino acids, such as seleno-methionine substitutions for methionine.

[0018]   The co-crystals of the invention generally comprise a crystalline Glucokinase polypeptide in association with one or more compounds at an allosteric site of the polypeptide. The association may be covalent or non-covalent.

Production of Polypeptides

[0019]   The native and mutated glucokinase polypeptides described herein may be isolated from natural sources or produced by methods well known to those skilled in the art of molecular biology. Expression vectors to be used may contain a native or mutated Glucokinase polypeptide coding sequence and appropriate transcriptional and/or translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY; and Ausubel et al., 1989, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY.

[0020]   A variety of host-expression vector systems may be utilized to express the Glucokinase coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the Glucokinase coding sequence; yeast transformed with recombinant yeast expression vectors containing the Glucokinase coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g. baculovirus) containing the Glucokinase coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosiac virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the glucokinase coding sequence; or animal cell systems. The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promotors such as pL of bacteriophage μ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used;

when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloingin in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35 S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the glucokinase coding sequence, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

Crystallization of Polypeptides and Characterization of Crystal Structure

[0021] The apo, derivative and co-crystals of the invention can be obtained by techniques well-known in the art of protein cystallography, including batch, liquid bridge, dialysis, vapor diffusion and hanging drop methods (see e.g. McPherson, 1982, *Preparation and Analysis of Protein Crystals*, John Wiley, NY; McPherson, 1990, *Eur. J. Biochem.* 189:1-23; Webber, 1991, *Adv. Protein Chem.* 41:1-36; Crystallization of Nucleic Acids and Proteins, Edited by Arnaud Ducruix and Richard Giege, Oxford University Press; Protein Crystallization Techniques, Strategies, and Tips, Edited by Terese Bergfors, International University Line, 1999). Generally, the apo- or co-crystals of the invention are grown by placing a substantially pure Gluckokinase polypeptide in an aqueous buffer containing a precipitant at a concentration just below that necessary to precipitate the protein. Water is then removed from the solution by controlled evaporation to produce crystallizing conditions, which are maintained until crystal growth ceases.

[0022] In a preferred embodiment of the invention, apo or co-crystals are grown by vapor diffusion. In this method, the polypeptide/precipitant solution is allowed to equilibrate in a closed container with a larger aqueous reservoir having a precipitant concentration optimal for producing crystals. Generally, less than about 10 μL of subtantially pure polypeptide solution is mixed with an equal volume of reservoir solution, giving a

precipitant concentration about half that required for crystallization. This solution is suspended as a droplet underneath a coverslip, which is sealed onto the top of a reservoir. The sealed container is allowed to stand, from one day to one year, usually for about 2-6 weeks, until crystals grow.

[0023] For crystals of the invention, it has been found that hanging drops containing about 2-5 μl of Glucokinase (9-22 mg/ml in 20 mM tris pH 7.1 measured at room temperature, 50 mM NaCl, 50 mM glucose, 10 mM DTT and optionally 0.2 mM EDTA) and an equal amount of reservoir solution (16-25% w/v polyethylene glycol with an average molecular weight from about 8000 to about 10000 Daltons, 0.1-0.2 M tris or bistris or Hepes or ammonium phosphate buffer, pH 6.9-7.5, 8-10 mM DTT, 0 - 30% saturated glucose) suspended over 0.5 to 1.0 mL reservoir buffer for about 3-4 weeks at 4-6°C provided crystals suitable for high resolution X-ray structure determination. Particularly preferred conditions were: about 2-5 μl of Glucokinase (10 mg/ml in 20 mM tris pH 7.1 measured at room temperature, 50 mM NaCl, 50 mM glucose, 10 mM DTT and optionally 0.2 mM EDTA) and an equal amount of reservoir solution (22.5% w/v polyethylene glycol with an average molecular weight of about 10000 Daltons, 0.1 M tris pH 7.08, 10 mM DTT, 20% glucose) were suspended over 0.5 to 1.0 mL reservoir buffer for about 3-4 weeks at 4-6°C.

[0024] The optimum procedure for growing crystals large enough to collect data from involved first streaking 3-4 μl of protein solution on the coverslip, followed by streaking 3-4 μl of well solution across the elongated droplet of protein, forming a droplet shaped like the letter 'X'. Before discovering this crossed droplet technique, most droplets yielded showers of small crystals which were not large enough for data collection purposes. The crossed droplets allow gradients of protein and precipitating agent to form as the two solutions slowly mix, and the resulting kinetics of crystal nucleation and growth are optimal for the growth of a small number of large crystals in each crossed droplet. Simply mixing the protein and precipitant solutions together in a single round droplet often produced an overabundance of nuclei which grew to a final size too small for data collection purposes. Crystals usually appeared within 5 days of setup. The

crystals grow in the form of hexagonal bipyramids, reaching dimensions of 0.2 x 0.2 x 0.4 mm typically, although larger crystals are often observed. Figure 1 shows grown crystals.

[0025] Crystals may be frozen prior to data collection. The crystals were cryo-protected with either (a) 20-30% saturated glucose present in the crystallization setup, (b) ethanol added to 15-20%, (c) ethylene glycol added to 10-20% and PEG10,000 brought up to 25%, or (d) glycerol added to 15%. The crystals were either briefly immersed in the cryo-protectant or soaked in the cryo-protectant for periods as long as a day. Freezing was accomplished by immersing the crystal in a bath of liquid nitrogen or by placing the crystal in a stream of nitrogen gas at 100 K.

[0026] The mosaic spread of the frozen crystals could sometimes be reduced by annealing, wherein the stream of cold nitrogen gas is briefly blocked, allowing the frozen crystal to thaw momentarily before re-freezing in the nitrogen gas stream. Another technique which was sometimes helpful in data collection was to center one of the ends of the hexagonal bipyramid in the x-ray beam, rather than the mid portion of the crystal. The mosaic spread could sometimes be reduced by this technique.

[0027] Diffraction data typically extending to 2.7 Å was collected from the frozen crystals at the synchrotron beamline X8C of the National Synchrotron Light Source in Brookhaven, New York. Under optimum conditions, data extending to 2.2 Å was recorded. See Figures 3 and 4 for solution. The space group of the crystals was determined to be P6(5)22 during the course of the solution of the crystal structure. The crystals have unit cell dimensions a = b = 79.62 +/- 0.60 Å, c = 321.73 +/- 3.70 Å, $\alpha = \beta$ = 90°, γ = 120°. The crystals are in a hexagonal system with P6(5)22 symmetry.

[0028] Of course, those having skill in the art will recognize that the above-described crystallization conditions can be varied. Such variations may be used alone or in combination, and include polypeptide solutions containing polypeptide concentrations between 1 mg/mL and 60 mg/mL, any commercially available buffer systems which can

maintain pH from about 6.5 to about 7.6, Tris-HCl concentrations between 10 mM and 200 mM, dithiothreitol concentrations between 0 mM and 20 mM, substitution of dithiothreitol with beta mercapto ethanol or other art-recognized equivalents, or substitution of glucose with other sugars known to bind to Glucokinase; and reservoir solutions containing polyethylene glycol concentrations between about 10% and about 30%, polyethylene glycol average molecular weights between about 1000 and about 20,000 daltons, any commercially available buffer systems which can maintain pH from about 6.5 to about 7.6, dithiothreitol concentrations between 0 mM and 20 mM, substitution of dithiothreitol with beta mercapto ethanol or other art-recognized -SH group containing equivalents, or substitution of glucose with other sugars known to bind to Glucokinase, and temperature ranges between 4 and 20°C.

[0029] Derivative crystals of the invention can be obtained by soaking apo or co-crystals in mother liquor containing salts of heavy metal atoms, according to procedures known to those of skill in the art of X-ray crystallography.

[0030] Co-crystals of the invention can be obtained by soaking an apo crystal in mother liquor containing a ligand that binds to the allosteric site, or can be obtained by co-crystallizing the Glucokinase polypeptide in the presence of one or more ligands that bind to the allosteric site. Preferably, co-crystals are formed with a glucokinase activator disclosed in US Pat. No. 6,320,050; US Pat. Appl. 09/532,506 filed March 21, 2000; US Pat. Appl. 09/675,781 filed September 28, 2000; US Pat. Appl. 09/727,624, filed December 1, 2000; US Pat. Appl. 09/841,983, filed April 25, 2001; US Pat. Appl. 09/843,466, filed April 26, 2001; US Pat. Appl. 09/846,820, filed May 1, 2001; US Pat. Appl. 09/846,821, filed May 1, 2001; US Pat. Appl. 09/905,152, filed July 13, 2001; US Pat. Appl. 09/924,247, filed August 8, 2001; US Provisional Pat. Appl. 60/251,637, filed December 6, 2000; or US Provisional Pat. Appl. 60/318,715, filed September 13, 2001, each of which is incorporated herein by reference.

[0031] Methods for obtaining the three-dimensional structure of the crystalline glucokinases described herein, as well as the atomic structure coordinates, are well-

known in the art (see, e.g., D. E. McRee, Practical Protein Crystallography, published by Academic Press, San Diego (1993), and references cited therein).

Uses of the Crystals and Atomic Structure Coordinates

[0032]   The crystals of the invention, and particularly the atomic structure coordinates obtained therefrom, have a wide variety of uses. For example, the crystals and structure coordinates described herein are particularly useful for identifying compounds that activate Glucokinases as an approach towards developing new therapeutic agents.

[0033]   The structure coordinates described herein can be used as phasing models in determining the crystal structures of additional native or mutated glucokinases, as well as the structures of co-crystals of such glucokinases with allosteric inhibitors or activators bound. The structure coordinates, as well as models of the three-dimensional structures obtained therefrom, can also be used to aid the elucidation of solution-based structures of native or mutated glucokinases, such as those obtained via NMR. Thus, the crystals and atomic structure coordinates of the invention provide a convenient means for elucidating the structures and functions of glucokinases.

[0034]   For purposes of clarity and discussion, the crystals of the invention will be described by reference to specific Glucokinase exemplary apo crystals and co-crystals. Those skilled in the art will appreciate that the principles described herein are generally applicable to crystals of any mammalian Glucokinase, including, but not limited to the Glucokinase of Figure 2.

DEFINITIONS

[0035]   As used herein, "allosteric site" refers in general to any ligand binding site on a mammalian Glucokinase other than the active site of the enzyme.

[0036] As used herein, "apo crystal" refers to crystals of mammalian Glucokinase formed without a bound allosteric ligand.

[0037] As used herein, "allosteric ligand" refers to any molecule which specifically binds an allosteric site on a mammalian Glucokinase.

EXAMPLES

Example 1: Expression and Purification of Glucokinase

Expression of GK

[0038] Glucokinase (GK) was expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli*. The amino-acid sequence of the fusion protein is given in Figure 2. The expression construct is based on the pGEX-3X vector from Pharmacia, as described in Y. Liang, P. Kesavan, L. Wang, K. Niswender, Y. Tanizawa, M. A. Permutt, M. A. Magnuson, F. M. Matschinsky, *Biochem. J.* **309**, 167 (1995). The construct codes for one of the two liver isozymes of human GK. The GST tag is at the N-terminus of the construct, and is separated from the coding sequence for GK by a Factor Xa cleavage site. After purification of the GST fusion protein, the GST fusion tag was removed with Factor Xa protease, which also removes five residues from the N-terminus of GK.

Purification of GK

[0039] *E. coli* cells expressing GST-GK were suspended in lysis buffer (50 mM tris, 200 mM NaCl, 5 mM EDTA, 5 mM DTT, 1% NP-40, pH 7.7) in the presence of protease inhibitors, incubated with lysozyme at 200 μ/ml for 30 minutes at room temperature, and sonicated 4x30 sec. at 4° C. After centrifugation to remove insoluble material, the supernatant was loaded onto glutathione-Sepharose, washed with lysis buffer and then with lysis buffer minus NP-40. GST-GK was eluted with lysis buffer (minus NP-40)

containing 50 mM D-glucose and 20 mM glutathione. The eluted protein was concentrated and dialyzed into 20 mM tris, 100 mM NaCl, 0.2 mM EDTA, 50 mM D-glucose, 1mM DTT, pH 7.7. Factor Xa was added at a protein ratio of 1:100 GST-GK followed by the addition of $CaCl_2$ to 1 mM, and the sample was incubated at 4° C for 48 hours. The sample was added to glutathione Sepharose and the unbound fraction collected and concentrated. The sample was then incubated with benzamidine Sepharose to remove Factor Xa, and the unbound fraction was collected and loaded on a Q Sepharose column equilibrated with 25 mM bis-tris propane, 50 mM NaCl, 5 mM DTT, 50 mM D-glucose and 5% glycerol (pH 7.0). The protein was eluted with a NaCl gradient from 50-400 mM. Fractions containing purified GK were pooled and concentrated and filtered.

Example 2: Formation of apo Crystal

[0040]   4 µl of glucokinase and 4 µl of precipitant were mixed and equilibrated against the precipitant solution at 4° C.   The glucokinase solution consisted of 22 mg/ml glucokinase prepared in Example 1 in 20 mM hepes pH 7.5, 50 mM NaCl, 10 mM DTT, and 50 mM glucose. The precipitant consisted of 22.5% PEG10000, 0.1 M tris pH 7.08, 10 mM DTT, 20% glucose; the precipitant solution contained seed crystals in order to microseed the droplets. Crystals appeared in the droplets after leaving the crystallization plates at 4° C.

Example 3: Formation of Co-crystal with 3-Cyclopentyl-2-pyridin-4-yl-N-thiazol-2-yl-propionamide

3(a):

[0041]   4 µl of glucokinase and 4 µl of precipitant were mixed and equilibrated against the precipitant solution at 4° C.   The glucokinase solution consisted of 13 mg/ml glucokinase prepared in Example 1 in 20 mM tris pH 7.0, 50 mM NaCl, 10 mM DTT, 50 mM glucose, and the glucokinase activator 3-Cyclopentyl-2-pyridin-4-yl-N-thiazol-2-yl-propionamide at a concentration 5 times that of the protein. The precipitant consisted of 22.5% PEG10000, 0.1 M tris pH 7.08, 10 mM DTT, 20% glucose. Crystals appeared in the droplets after leaving the crystallization plates at 4° C.

3(b):

[0042] Alternatively, crystals were grown as in Example 3(a) with the following changes: instead of 4 μl glucokinase and 4 μl precipitant, 2 μl of each were used; the glucokinase solution contained 11 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 22.5% PEG10000 as precipitant 18% PEG8000 was used; the precipitant solution contained seed crystals in order to microseed the droplets.

3(c):

[0043] In another alternative, crystals were grown as in Example 3(a) with the following changes: instead of 4 μl glucokinase and 4 μl precipitant, 2 μl of each were used; the glucokinase solution contained 11 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 22.5% PEG10000 as precipitant 20% PEG8000 was used; the precipitant solution contained seed crystals in order to microseed the droplets.

3(d):

[0044] In yet another alternative, crystals were grown as in Example 3(a) with the following changes: instead of 4 μl glucokinase and 4 μl precipitant, 2 μl of each were used; the glucokinase solution contained 12 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 22.5% PEG10000 as precipitant 16% PEG10000 was used; glucose was not present as a component of the precipitant; the precipitant solution contained seed crystals in order to microseed the droplets.

3(e):

[0045] In still another alternative, crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 11 mg/ml glucokinase in tris buffer at pH 7.1 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 22.5% PEG10000 as precipitant 25% PEG10000 was used.

3(f):

[0046] In still another alternative, crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 11 mg/ml glucokinase in tris buffer at pH 7.1 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 22.5% PEG10000 as precipitant 21.25% PEG10000 was used; in place of tris buffered at pH 7.08 in the precipitant tris buffered at pH 7.52 was used.

3(g):

[0047] In still another alternative, crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 12 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of tris buffered at pH 7.08 in the precipitant, hepes buffered at pH 6.89 was used; in place of 20% glucose in the precipitant, 200 mM glucose was used.

3(h):

[0048] In still another alternative, crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 12 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 0.1 M tris buffered at pH 7.08 in the precipitant, 0.2 M ammonium phosphate buffered at pH 7.03 was used; in place of 20% glucose in the precipitant, 200 mM glucose was used.

3(i):

[0049] In still another alternative, crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 10 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 22.5% PEG10000 as precipitant, 20% PEG10000 was used; in place of tris buffered at pH 7.08 in the precipitant, tris buffered at pH 7.05 was used; in place of 10 mM DTT in the precipitant, 8 mM DTT was used; glucose was not present as a component of the precipitant.

3(j):

[0050] In still another alternative, crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 12 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 22.5% PEG10000 as precipitant, 22% PEG8000 was used; glucose was not present as a component of the precipitant; the precipitant solution contained seed crystals in order to microseed the droplets.

3(k):

[0051] In still another alternative, crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 11 mg/ml glucokinase in tris buffer at pH 7.1 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of 20% glucose in the precipitant, 30% glucose was used.

Example 4: Formation of Co-crystal with N-(5-Bromo-pyridin-2-yl)-2-(3-chloro-4-methanesulfonyl-phenyl)-3-cyclopentyl-propionamide

[0052] Crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 9 mg/ml glucokinase in tris buffer at pH 7.1 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of the glucokinase activator of Example 3(a), the glucokinase solution contained the glucokinase activator N-(5-Bromo-pyridin-2-yl)-2-(3-chloro-4-methanesulfonyl-phenyl)-3-cyclopentyl-propionamide; in place of 20% glucose in the precipitant, 200 mM glucose was used.

Example 5: Formation of Co-crystal with 2-(3-Chloro-4-methanesulfonyl-phenyl)-3-cyclopentyl-N-(5-trifluoromethyl-pyridin-2-yl)-propionamide

[0053] Crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 10 mg/ml glucokinase in tris buffer at pH 7.1 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of the glucokinase activator of Example 3(a), the glucokinase solution contained the glucokinase activator 2-

(3-Chloro-4-methanesulfonyl-phenyl)-3-cyclopentyl-N-(5-trifluoromethyl-pyridin-2-yl)-propionamide; in place of 22.5% PEG10000 as precipitant, 21.25% PEG10000 was used.

Example 6: Formation of Co-crystal with (2S)-2-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionylamino]-thiazole-4-carboxylic acid methyl ester

[0054] Crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 10 mg/ml glucokinase in tris buffer at pH 7.1 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of the glucokinase activator of Example 3(a), the glucokinase solution contained the glucokinase activator (2S)-2-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionylamino]-thiazole-4-carboxylic acid methyl ester; in place of 22.5% PEG10000 as precipitant, 21.25% PEG10000 was used; in place of tris buffered at pH 7.08 in the precipitant, bistris buffered at pH 7.0 was used.

Example 7: Formation of Co-crystal with (2S)-{2-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionylamino]-thiazol-5-yl}-oxo-acetic acid ethyl ester

[0055] Crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 10 mg/ml glucokinase in tris buffer at pH 7.1 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of the glucokinase activator of Example 3(a), the glucokinase solution contained the glucokinase activator (2S)-{2-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionylamino]-thiazol-5-yl}-oxo-acetic acid ethyl ester; in place of 22.5% PEG10000 as precipitant, 21.25% PEG10000 was used.

Example 8: Formation of Co-crystal with (2S)-{3-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionyl]-ureido}-acetic acid methylester

[0056] Crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 9 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of the glucokinase

activator of Example 3(a), the glucokinase solution contained the glucokinase activator (2S)-{3-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionyl]-ureido}-acetic acid methylester; in place of 20% glucose in the precipitant, 200 mM glucose was used.

Example 9: Formation of Co-crystal with (2S)-1-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionyl]-3-(3-hydroxy-propyl)-urea

[0057] Crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 14 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of the glucokinase activator of Example 3(a), the glucokinase solution contained the glucokinase activator (2S)-1-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionyl]-3-(3-hydroxy-propyl)-urea; in place of 20% glucose in the precipitant, 200 mM glucose was used.

Example 10: Formation of Co-crystal with (2S)-{3-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionyl]-ureido}-acetic acid ethyl ester

[0058] Crystals were grown as in Example 3(a) with the following changes: the glucokinase solution contained 14 mg/ml glucokinase in tris buffer at pH 7.08 instead of 7.0; the glucokinase solution included 0.2 mM EDTA; in place of the glucokinase activator of Example 3(a), the glucokinase solution contained the glucokinase activator (2S)-{3-[3-Cyclopentyl-2-(3,4-dichloro-phenyl)-propionyl]-ureido}-acetic acid ethyl ester; in place of tris buffered at pH 7.08 in the precipitant, tris buffered at pH 7.05 was used.

Example 11: Synthesis of 3-Cyclopentyl-2-pyridin-4-yl-N-thiazol-2-yl-propionamide

[0059] 3-Cyclopentyl-2-pyridin-4-yl-N-thiazol-2-yl-propionamide can be prepared using well-known organic synthesis techniques according to the following reaction scheme:
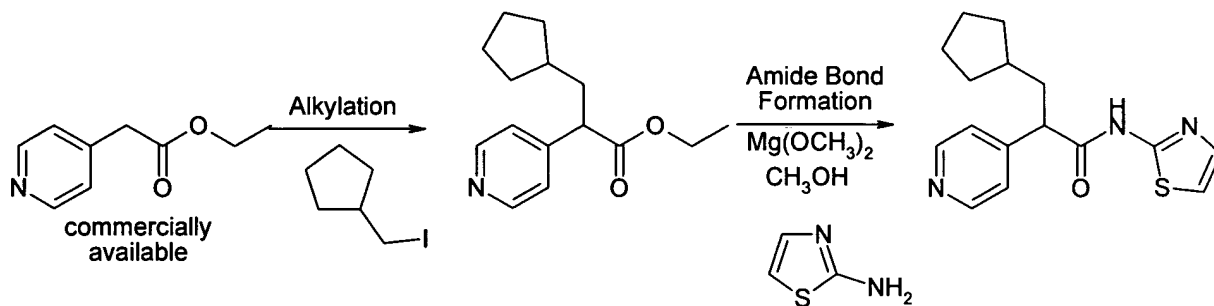
[0060] 3-Cyclopentyl-2-pyridin-4-yl-N-thiazol-2-yl-propionamide is useful as an allosteric activator of Glucokinase and to assist the formation of co-crystals of Glucokinase.